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## Effector ExoU from the Type III Secretion System Is an Important Modulator of Gene Expression in Lung Epithelial Cells in Response to *Pseudomonas aeruginosa* Infection

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*Pseudomonas aeruginosa* is an important pathogen in immunocompromised patients and secretes a diverse set of virulence factors that aid colonization and influence host cell defenses. An important early step in the establishment of infection is the production of type III-secreted effectors translocated into host cells by the bacteria. We used cDNA microarrays to compare the transcriptomic response of lung epithelial cells to *P. aeruginosa* mutants defective in type IV pili, the type III secretion apparatus, or in the production of specific type III-secreted effectors. Of the 18,000 cDNA clones analyzed, 55 were induced or repressed after 4 h of infection and could be classified into four different expression patterns. These include (i) host genes that are induced or repressed in a type III secretion-independent manner (32 clones), (ii) host genes induced specifically by ExoU (20 clones), and (iii) host genes induced in an ExoU-independent but type III secretion dependent manner (3 clones). In particular, ExoU was essential for the expression of immediate-early response genes, including the transcription factor c-Fos. ExoU-dependent gene expression was mediated in part by early and transient activation of the AP1 transcription factor complex. In conclusion, the present study provides a detailed insight into the response of epithelial cells to infection and indicates the significant role played by the type III virulence mechanism in the initial host response.

*Pseudomonas aeruginosa* is one of the most virulent opportunistic pathogens in humans. In the setting of preexisting epithelial injury, particularly in immunocompromised patients, it leads to devastatingly acute infections, including pneumonia and sepsis (52). Up to 75% of patients in intensive care units are colonized with *P. aeruginosa*, and the mortality from pneumonia is 40%, even with antibiotic treatment (39).

*P. aeruginosa* boasts an impressive array of cell-associated and secreted virulence factors that contribute to its pathogenesis. Key among these is type IV pili, the major bacterial adhesin, and the type III secretion system with its secreted exotoxins. Type IV pili are polar fimbriae comprised of pilin, the product of the pilA gene (reviewed in reference 40). A unique feature of type IV pili is their ability to extend and retract (41). In addition to their roles in adhesion, type IV pili are also involved in biofilm formation (55) and twitching motility (63) and serve as bacteriophage receptors (49). They are required for full virulence in a mouse model of acute pneumonia (3, 11).

Upon host cell contact, the type III secretion system allows bacteria to directly inject toxins into the host cell, where they subvert host cell defense and signaling systems (24). Four type III-secreted effectors have been identified in *P. aeruginosa*, although few if any strains secrete all four of them (reviewed in reference 9). PA103, a human lung isolate, encodes and se-

cretes two effectors, ExoU and ExoT. ExoU is a potent cytotoxin whose host cell targets and mechanism of action are not yet known (12, 13, 21). ExoT is a bifunctional protein, possessing an N-terminal GTPase-activating domain with GAP activity toward Rho, Rac, and Cdc42, and a C-terminal ADP-ribosyltransferase domain (16, 32, 35). The presence of a functional type III secretion system is associated with poor outcome in acute *P. aeruginosa* infections in humans (19, 51).

Evidence also suggests that type III effectors, in addition to their effects on host cellular enzymes, are likely to evoke rapid and specific changes in gene expression. Peripheral blood mononuclear cells respond to purified ExoS exposure by increasing the transcription of proinflammatory cytokines and chemokines (10, 34), and macrophages upregulate numerous genes in response to effectors of the homologous *Yersinia enterocolitica* type III secretion system (53). The lung epithelium, which represents an important barrier to infection and a primary target of type III-secreted effectors, responds to *P. aeruginosa* infection through the expression of many genes involved in host defense and immune cell activation (25). However, the role played by type III secretion in this response is not known. Here we use microarray analysis and well-characterized mutant strains of cytotoxic *P. aeruginosa* to investigate the response of lung epithelial cells to different type III effectors. More than 18,000 genetic elements were analyzed, and this resulted in the classification of expression patterns both dependent and independent of the type III toxin function. The type III-independent response consisted of genes involved in inflammatory responses and cytoprotective roles, whereas the majority of type III-dependent genes, induced through the

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TABLE 1. Strains used in this study

Strain	Relevant characteristic(s) <sup>a</sup>	Reference
PA103	Virulent lung isolate of <i>P. aeruginosa</i> ; known type III secreted effector proteins are ExoT and ExoU	1, 38
PA103 <i>exoT</i>	PA103 with an <i>xylE aacC1</i> cassette replacing aa 36 to 348 of <i>exoT</i> ; Gm <sup>r</sup>	16
PA103 <i>exoU</i>	PA103 with an in-frame deletion of aa 330 to 571 of <i>exoU</i>	16
PA103 <i>exoT exoU</i>	PA103 <i>exoU</i> with an <i>xylE aacC1</i> cassette replacing aa 36 to 348 of <i>exoT</i> ; Gm <sup>r</sup>	16
PA103 <i>pscJ::Tn5</i>	Tn5 Gm <sup>r</sup> cassette inserted into <i>pscJ</i> ; defective in type III secretion	29
PA103 <i>pilA</i>	PA103 with <i>StuI/Bsu36I</i> region of <i>pilA</i> replaced with a Gm <sup>r</sup> cassette	4

<sup>a</sup> aa, amino acids; Gm<sup>r</sup>, gentamycin resistance.

action of either ExoU or other unidentified type III effectors, were transcriptional or cell signal regulators. We also show that the mechanism by which ExoU induced gene expression involves the early and transient activation of the AP1 transcription factor complex.

## MATERIALS AND METHODS

**Bacteria, cells, and infection protocol.** *P. aeruginosa* strain PA103, a cytotoxic lung isolate, and mutants derived from this strain that were used in the present study are described in Table 1. Two days prior to use, bacteria were inoculated from glycerol stocks onto Luria-Bertani (LB) agar plates and grown overnight at 37°C, and single colonies were subsequently incubated overnight in 2 ml of LB broth at 37°C without shaking. The 9HTEo<sup>−</sup> cells are a simian virus 40 T-antigen-transformed line derived from human tracheal epithelium and were kindly provided by D. Gruenert (University of Vermont). The cells were maintained in Dulbecco modified Eagle medium supplemented with glutamine (2 mM; Gibco), streptomycin (50 µg/ml; Gibco), penicillin (50 U/ml; Gibco), and Serum Supreme (10%; BioWhittaker). The cells were grown on plastic vessels precoated with a solution containing Vitrogen (0.2 mg/ml; Cohesion), NaOH (0.83 mM), and Ham F-12 medium (10%; Gibco). For infection studies, cells (between passages 18 to 22) were seeded at 150,000 cells/cm<sup>2</sup>, grown until completely confluent (usually 3 days), and then washed and incubated in serum- and antibiotic-free medium for a further 20 h. Freshly grown bacteria in LB broth were diluted into the serum- and antibiotic-free medium and added to the cells at a multiplicity of ca. 10 bacteria/cell. The amount of bacterial inoculum was enumerated by serial dilution and plating on LB agar plates.

Care was taken to use completely confluent monolayers of 9HTEo<sup>−</sup> cells in the infection studies since they become polarized, form tight junctions (18), and resist the cytotoxic effects of PA103 up to 5 h after infection (B. McMorran, unpublished observations).

**Microarray hybridization and analysis.** Total RNA was prepared from infected and uninfected (control) cells by using the RNeasy RNA isolation kit (Qiagen). RNA samples (40 µg) were labeled with Cy5 or Cy3 dUTP, purified, and subjected to microarray hybridization as described previously (26). Equal amounts of control (noninfected, Cy3-labeled) and infected (Cy5-labeled) sample were hybridized to microarray chips (human 19K2.2; Ontario Cancer Institute) overnight at 45°C in humidified chambers. Slides were washed for 3 min in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.05% sodium dodecyl sulfate (SDS) and then twice for 3 min each time in 0.2× SSC prior to obtaining fluorescence images with a Genetic MicroSystems G418 scanner.

Spot intensities were quantified by using Image software (Biodiscovery). The proportion of “passed” spots (i.e., with detectable intensity) on each array was 60 to 70%, and no intrachip or spatial variations in hybridization signals were observed. Quantified data were normalized and analyzed by using the GeneSpring package (Silicon Genetics). For normalization the 40th percentile of all measurements was used as a positive control for each sample; each measurement for each gene was divided by this synthetic positive control, assuming that this was at least 0.01. The bottom tenth percentile was used as a test for correct background subtraction. To calculate the presented ratios, the measured intensity of each gene was divided by its control channel value. When the control channel intensity was <200, the datum point was considered unreliable and omitted. Raw data are viewable at <http://microarray.imb.uq.edu.au/base/www/index.phtml> (login: guest-wainwright; password: XuFEG1D2).

Clones corresponding to spots of interest were obtained from the Ontario Cancer Institute. Plasmids from pure cultures were sequenced with M13 primers (22), and identity was established by using the basic local alignment search tool (BLAST) and the NCBI GenBank database. Plasmids were digested to liberate

insert without poly(A)<sup>+</sup> tail, where possible, and separated on an agarose gel with the QiaQuick gel extraction kit (Qiagen) to be used as Northern probes.

**Northern blotting.** Total RNA (15 µg) was separated on a 1% agarose-formaldehyde gel and transferred to Magna nylon membrane (Osmonics). Pre-hybridization (3 h) and hybridization (overnight) were performed in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, and 1 mM EDTA [pH 7.7]), 5× Denhardt solution, 0.5% SDS, 1 µg of heat-denatured sheared salmon sperm DNA/ml (Sigma), and 50% formamide at 42°C. Probes were labeled by using [ $\alpha$ -<sup>32</sup>P]CTP and a random priming (Megaprime; Amersham Pharmacia) and then purified on G-50 Sephadex. Hybridized blots were washed at high stringency (0.1× SSC–0.1% SDS at 42°C). Bands were detected and quantified by phosphorimaging with phosphor screens (Kodak) and a Storm scanner and ImageQuant software (both from Molecular Dynamics). A 600-bp fragment of the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA was used a loading control in the quantification calculations.

**Immunofluorescence staining.** Cells for staining studies were grown on Vitrogen-coated glass coverslips and, after infection, were washed in phosphate-buffered saline (with three 2-min washes) and fixed in cold methanol (30 min). Staining was performed on permeabilized cells (0.1% Triton X-100–phosphate-buffered saline for 10 s) in a solution containing 0.5% bovine serum albumin (Sigma). The primary antibodies and dilutions used were anti-cFos at 1/100 (clone 4-G, goat polyclonal immunoglobulin G [IgG]; Santa Cruz Biotechnology) and anti-*P. aeruginosa* at 1/400 (purified rabbit sera [a gift from C. Whitchurch, University of California at San Francisco]). Secondary antibodies, Cy3-labeled donkey anti-goat IgG, and Alexa Fluor 488-labeled chicken anti-rabbit IgG (Molecular Probes) were used at a 1/400 dilution. Fluorescence staining was visualized on an Olympus AX-70 microscope with a ×60 oil immersion objective lens. Images were captured from a CCD300-RC charge-coupled device camera (Dage-MTI) by using NIH Image 1.62 software and were merged and processed by using Adobe Photoshop 5.0.2.

**Electrophoretic mobility shift assay (EMSA).** Preparation of the nuclear extracts, probe preparation and binding reactions were performed essentially as described previously (56). Extracts were prepared from ca. 1.2 × 10<sup>7</sup> 9HTEo<sup>−</sup> cells. An AP1-specific double-stranded oligonucleotide (CGATTGACTCAGT ACTGAGTCAATCG, with the consensus AP1-binding sites underlined) and a nonspecific oligonucleotide of similar size were used in the binding reactions. For the antibody inhibition experiments, 3 µg of anti-cFos or anti-β-galactosidase (goat polyclonal IgG; Cortex Biochem) antibody was included in the binding reaction. Protein-DNA complexes were separated on 6% nondenaturing polyacrylamide gels (acrylamide-bisacrylamide [29:1]) run at 100 V in 0.5× Tris-borate-EDTA buffer. The gels were dried and visualized by autoradiography.

## RESULTS

**Microarray analysis identifies genes regulated in 9HTEo<sup>−</sup> cells by *P. aeruginosa* infection.** To assess and compare the contributions of type III secretion to the transcriptional host cell response, *exoT*, *exoU*, *exoT exoU*, *pscJ*, and *pilA* isogenic mutants and the parental wild-type PA103 strains of *P. aeruginosa* were used (Table 1). The former three strains harbor deletions in the ExoU and/or ExoT genes and do not secrete the respective toxins, although they possess a functional type III secretion apparatus. The *pscJ* mutant harbors a transposon insertion in the *pscJ* gene and has a nonfunctional type III secretion apparatus (29). The *pilA* strain contains an antibiotic resistance cassette in the pilin gene and lacks type IV pili; it is

also defective for type III-dependent effects (29; Jakobsen and J. Engel, unpublished results). Cells were infected for 4 h and subsequently subjected to microarray analysis. cDNA was prepared from cells infected with each strain (Cy5 labeled), pooled with cDNA from uninfected cells as a common reference (Cy3 labeled), and hybridized to microarrays containing over 18,000 cDNA clones (with ca. 25% redundancy) (Ontario Cancer Institute, Toronto, Ontario, Canada). The microarrays contained duplicate spots of each cDNA clone, providing two measurements of relative gene expression. In addition, duplicate hybridizations were performed by using independently grown and infected cell material (biological replicates) to allow for technical and biological variation between hybridizations. Raw duplicate data sets were merged into one experiment by using Genespring software (Silicon Genetics) and subjected to normalization and expression analysis as described in Materials and Methods.

We first sought to identify genes that were regulated by infection by comparing expression levels of PA103 wild-type infected and noninfected cells. Representative scatter plots for noninfected/noninfected and parental/noninfected hybridizations are illustrated in Fig. 1. Extremely low variation is evident in the first plot, indicating that the Cy3 and Cy5 incorporation and hybridization efficiencies were similar. In the second plot, the majority of genes lie within a twofold ratio range and were considered unregulated by infection. Datum points located outside the twofold boundaries were taken to be significantly different from control expression and chosen for further analysis.

A total of 9,243 cDNA elements were used in the analysis, and 55 clones, representing ca. 46 unique genes, were significantly and reproducibly up- or downregulated by *P. aeruginosa* infection compared to noninfected cells (Table 2). These genes represent the total transcriptional response to infection measured by the arrays. Each of the clones was independently sequenced to verify its identity on the array. Although this is a relatively small proportion of the total number analyzed, an initial examination indicated several upregulated genes previously shown as inducible by *P. aeruginosa* infection. These genes included monocyte chemotactic protein (MCP1/SCYA2), tristetraproline (TTP), ras homolog RhoB, viral oncogene homolog *c-fos* and urokinase type plasminogen activator receptor (PLAUR) genes (25). Raw expression intensity values were also very similar between the different samples, indicating that the changes were due to infection and not to variation in the reference signal. Northern blots performed on RNA from additional infection experiments were used to check the reproducibility of the array data and were in close agreement with expression patterns (see Fig. 3).

**Identification of genes regulated dependently and independently by type III-secreted toxins.** Cluster analyses allowed the infection-regulated genes to be categorized into one of four expression pattern classes (Fig. 2): (i) upregulated by infection with all six strains (class A); (ii) downregulated by all six strains (class B); (iii) upregulated only by the parental and *exoT* mutant strains (class C); and (iv) upregulated only by the parental and *exoT*, *exoU*, and *exoT exoU* mutant strains (class D). Since the first two classes of genes were regulated equivalently by all of the strains including parental, we termed this group type III-independent genes (Table 2). Class C genes were only

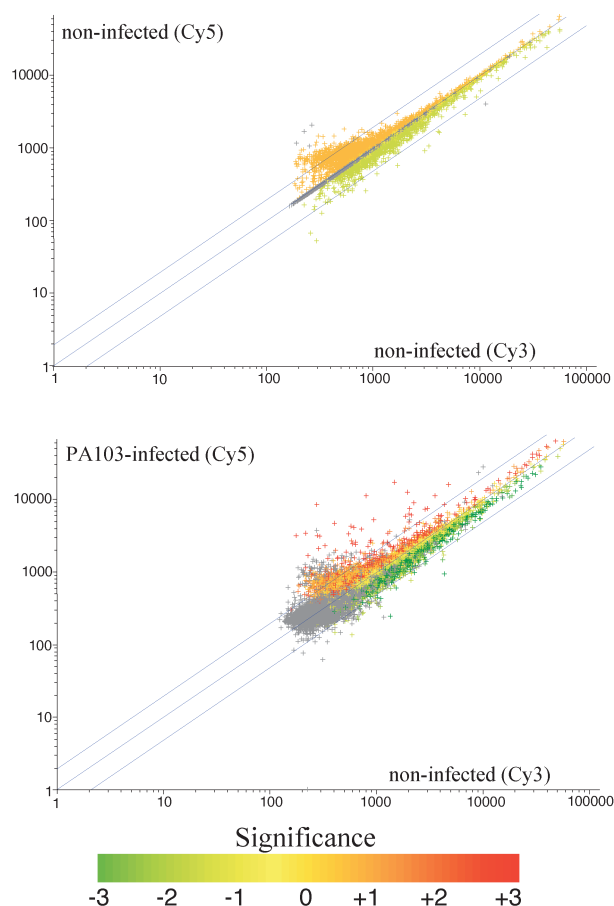


FIG. 1. Scatterplot profiles of 9HTEo- cell genes after microarray hybridization and normalization. Each plot represents the Cy3 versus Cy5 expression intensity ratios of 9,243 genes derived from microarray hybridization experiments comparing noninfected and noninfected cells (top) or comparing noninfected and PA103-infected cells (bottom). Genes which lie outside the upper and lower twofold boundaries (blue lines) are significantly up- or downregulated in infected versus noninfected cells. The significance of these expression changes are indicated by graded coloring from one, two, or three standard deviations difference between infected and noninfected intensities. Gray color refers to genes lacking duplicate datum points.

induced by strains able to secrete ExoU, so this group was termed ExoU dependent (Table 2). Class D genes were induced by mutants with specific deletions in ExoU and ExoT, as well as the parental strain, but were not regulated by the mutants completely defective in type III secretion. Therefore, this group was termed type III secretion dependent (Table 2).

Approximately 23 unique genes (represented by 27 clones) were induced (class A) and 5 were repressed (class B) in a type III secretion-independent manner. The products of these genes were classified according to their major cellular functions. The majority were involved in either the control of immune and host defense mechanisms ( $n = 5$ ) or oxidant protection ( $n = 4$ ). Molecules with roles in transcriptional regulation ( $n = 2$ ), cell signaling ( $n = 4$ ), and solute transportation ( $n = 2$ ) were also represented, along with five uncharacterized expressed sequence tags (ESTs). Northern blots on a selection of these genes confirmed that these expression pat-



TABLE 2. Relative expression ratios of 9HTEo- cell genes regulated by *P. aeruginosa* infection

Class and gene designation	Full gene name	Accession no. <sup>a</sup>	Function	Relative expression ratio of <i>P. aeruginosa</i> strain:						<i>p</i> <sup>b</sup>
				PA103	<i>exoT</i>	<i>exoU</i>	<i>exoTU</i>	<i>pscJ</i>	<i>pilA</i>	
Class A: type III independently activated genes										
VEGFA	Vascular endothelial growth factor A	n91060	Proinflammatory	8.04	4.98	5.65	5.72	5.69	6.17	0.001
VEGFA	Vascular endothelial growth factor A	aa126903	Proinflammatory	4.9	4.46	2.76	2.65	3.86	3.37	0.001
IGFBP3	Insulin-like growth factor binding protein 3	n24118	Proinflammatory	2.58	2.02	2.71	2.58	3.65	2.91	0.1
ADM	Adrenomedullin	w19284	Proinflammatory, antimicrobial	6.43	2.15	9.62	4.97	5.76	5.43	0.1
FSTL3	Follistatin-like 3	n75101	Proinflammatory	2.13	1.78	1.63	2.4	2.18	1.59	0.04
IRS2	Insulin receptor substrate-2	aa035325	Signal regulator	3.41	1.47	2	2.31	2.57	2.05	0.01
ANKRD3, DIK	Dual-specificity Ser/Thr/Tyr kinase 3 ( <i>dik</i> gene)	w37332	Signal regulator	3.71	2.63	4.66	3.49	4.41	3.59	0.06
STC1S	Stanniocalcin 1	r48681	Autocrine signal	20.37	4.75	11.75	11.22	13.66	6.23	0.08
SRA1	Steroid receptor RNA activator	r50254	Transcription regulator	7.34	4.34	6.49	6.57	8.44	9.68	0.08
NFIL3	Nuclear factor regulated by IL-3	w67706	Transcription activator	5.59	4.69	4.85	4.74	7.56	3.99	0.03
MT1E	Metallothionein 1E	h93127	Oxidant protection	4.64	1.61	4.05	2.06	4.68	3.42	0.06
MT1E	Metallothionein 1E	h52525	Oxidant protection	3.47	1.43	3.87	2.49	4.4	4.06	0.17
MT1G	Metallothionein 1G	w78010	Oxidant protection	4.3	1.37	3.08	3.25	4.1	3.71	0.15
MT1G	Metallothionein 1G	h57208	Oxidant protection	2.9	1.55	2.7	2.52	4.22	4.13	0.2
MT2A	Metallothionein 2A	h91612	Oxidant protection	4.22	1.54	2.95	2.91	3.81	4.08	0.2
Oatprp1	Organic anion transporter member 12	h84604	Membrane transport	3.05	2.33	1.85	5.51	2.7	3.12	0.2
SLC2A1	Solute carrier protein 2	w58375	Membrane transport	2.96	3.66	2.35	3.18	2.93	2.66	0.02
ADFP	Adipose differentiation-related protein (adipophilin)	w20444	Membrane protein, inducible	3.42	2.51	1.94	1.69	2.87	2.01	0.2
U2AF65	U2 small nuclear ribonucleoprotein auxiliary factor	aa053859	mRNA processing	2.73	1.64	1.94	1.89	1.42	1.45	0.01
RNA pol I (16 kDa)	RNA polymerase I 16-kDa subunit	n71041	mRNA processing	2.28	1.72	1.58	1.83	2.01	1.63	0.2
PCR17	Rab GTPase-activating protein	aa047487	Signal regulator	2.25	0.94	2.07	2.53	1.91	1.7	0.2
SYN2	Syndecan 2	aa156696	Wound repair	3.1	2.43	3.83	3.37	3.68	2.6	0.1
EHD4	EH domain containing 4	n94535		2.28	1.48	2.52	1.29	2.15	2.53	0.2
EST	Chromosome 14q24.3, BAC 201F1	h91088		3.52	3.34	3.21	2.9	3.09	2.89	0.01
EST	Hypothetical protein FLJ21616	r98518		3.03	1.33	2.54	2.33	3.33	3.68	0.07
EST	No homology	n68160		3.01	2.68	2.69	3.33	1.94	1.96	0.17
EST	Hypothetical protein DKFZp566J091	aa040473		2.91	1.9	2.09	2.15	2.19	2.26	0.02
Class B type III independently repressed genes										
DUSP6	Dual-specificity phosphatase 6	t65557	Signal regulator	0.65	0.39	0.46	0.59	0.56	0.54	0.17
TRXIP	Thioredoxin-interacting protein	n71361	Oxidant protection	0.24	0.26	0.15	0.13	0.15	0.15	0.001
EST	KIAA1515 protein	r40307		0.24	0.15	0.079	0.073	0.13	0.08	0.1
EST	Chromosome 11q, clone:RP11-687M24	h09816		0.35	0.7	0.49	0.66	0.53	0.64	0.003
EST	Hypothetical protein (HSPC210)	h72619		0.43	0.06	0.43	0.12	0.17	0.16	0.1
Class C: <i>exoU</i> -dependent activated genes										
DUSP1	Dual-specificity phosphatase 1	r79387	Signal regulator	50.19	13.76	3.97	3.15	2.16	2.49	0.09
DUSP1	Dual-specificity phosphatase 1	h87493	Signal regulator	11.43	11.52	3.86	6.25	2.18	2.61	0.005
DUSP1	Dual-specificity phosphatase 1	h29136	Signal regulator	15.85	5.48	4.22	3.85	1.91	2.12	0.001
DUSP1	Dual-specificity phosphatase 1	h01773	Signal regulator	14.36	8.64	4.45	6	2.02	2.89	0.03
KNSL5	Kinesin-like 5	aa043507	Signal regulator	7.07	6.34	1.98	2.05	3.54	2.32	0.003
RhoB	Transforming protein RhoB ( <i>ras</i> homolog)	w67471	Signal regulator	7.3	0.668	2.85	2.06	2.46	1.41	0.1

Continued on following page

TABLE 2—Continued

Class and gene designation	Full gene name	Accession no. <sup>a</sup>	Function	Relative expression ratio of <i>P. aeruginosa</i> strain:						<i>p</i> <sup>b</sup>
				PA103	<i>exoT</i>	<i>exoU</i>	<i>exoTU</i>	<i>pscJ</i>	<i>pilA</i>	
RhoB	Transforming protein RhoB ( <i>ras</i> homolog)	r32081	Signal regulator	4.54	4.82	2.01	2.78	2.32	2.22	0.07
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	aa019816	Transcription activator	20.8	3.1	2.72	4.01	4.3	1.62	0.1
MYC	v-myc proto-oncogene protein	w87861	Transcription activator	10.37	5.66	3.42	2.44	5.95	3.69	0.1
MYC	v-myc proto-oncogene protein	h43827	Transcription activator	7.73	10	2.99	3.28	2.63	3.26	0.02
NR4A1/nur77	Nuclear receptor subfamily 4, grpA, murine nur77 homolog	n64388	Transcription activator	5.37	1.6	1.26	2.02	1.56	1.04	0.2
BPTF	Bromodomain transcription factor	h02279	Transcription activator	4.04	3.01	1.96	2.95	1.5	1.68	0.03
TTP	Tristetraproline	aa054080	Transcription activator mRNA stability	3.7	1.51	2.02	2.78	1.18	1.6	0.1
TCF8/	Transcription factor 8	aa150750	Transcription repressor	16.46	5.23	3	2.99	1.78	1.49	0.02
TCF8	Transcription factor 8	r43502	Transcription repressor	4.44	3.17	2.07	2.43	1.34	1.61	0.03
PLAUR	Urokinase type plasminogen activator receptor	t75241	Protease regulator	4.85	1.4	0.69	0.9	0.51	0.61	0.1
EST	Chromosome 14, BAC R-156E22	r85513		20.4	20.8	2.79	2.9	2.16	1.62	0.004
EST	Similar to hypothetical protein FLJ11328	aa044730		6.3	6.98	2.22	2.46	2.9	2.41	0.1
EST	No homology	h87673		4.97	3.65	1.55	1.71	1.14	1.74	0.09
EST	Hypothetical protein FLJ22182	aa156747		4.47	5.49	2.02	1.87	2.2	2	0.05
Class D: type III-dependent activated genes										
MCPI/SCYA2	Monocyte chemotactic protein 1	aa024753	Proinflammatory	1.93	2.66	3.63	3.78	0.72	1.31	0.1
MCPI/SCYA2	Monocyte chemotactic protein 1	r75975	Proinflammatory	1.12	2.32	2.44	2.88	0.7	0.82	0.1
TIEG	TGF-early inducible gene	aa045730	Transcription activator	2.62	2.21	1.87	1.97	1.35	1.21	0.002

<sup>a</sup> GenBank accession number.<sup>b</sup> As determined by Student *t* test analysis.

tern classes were reproducible. Adrenomedullin (ADM) was strongly induced as early as 3 h after infection and to equivalent levels by all strains (Fig. 3A), a finding consistent with the array results in Table 2. Genes downregulated by infection

were also confirmed in Northern blotting experiments. As an example, expression of thioredoxin-interacting protein (TRXIP) was very high in untreated cells but strongly repressed between 3 and 4 h after infection (Fig. 3B).

Approximately 14 genes (represented by 20 cDNA clones) fell into the class C (ExoU-dependent expression pattern; Table 2). Genes in this group were consistently expressed at least twofold higher in the ExoU-sufficient parental and *exoT* strains compared to the ExoU-deficient mutants (i.e., the *exoU*, *exoT* *exoU*, *pscJ*, and *pilA* mutants). In many cases, induction by the latter four strains was completely absent. The vast majority of characterized ExoU-dependent genes are involved in transcriptional and cell signal regulation ( $n = 9$ ). A further four clones represent unidentified ESTs. ExoU-dependent regulation of several of these genes was confirmed by Northern blotting. Transcripts encoding dual-specificity phosphatase 1-mitogen-activated protein (MAP) kinase phosphatase 1 (DUSP1/MKP1), nuclear orphan receptor 4A1 (NR4A1/nur77), and *c-fos* were all induced within 3 h of infection only by wild-type and *exoT* mutant strains (Table 2 and Fig. 3C to E). The expression pattern of RhoB suggested partial dependence on ExoU since all six strains induced the gene, but in the wild-type bacteria and ExoT mutant, induction was at least three times higher (Fig. 3F). We also found that interleukin-6

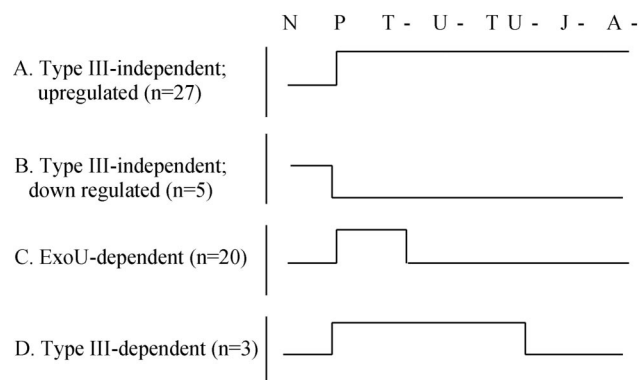


FIG. 2. Expression patterns of 9HTEo- cell genes regulated after *P. aeruginosa* infection. A total of 55 genes that showed >2-fold difference between noninfected cells (N) and cells infected by *P. aeruginosa* PA103 parental (P), *exoT* (T-), *exoU* (U-), *exoT* *exoU* (TU-), *pscJ* (J-), and *pilA* (A-) were clustered into four different expression profile classes (A to D). The number of genes in each group is indicated in parentheses.

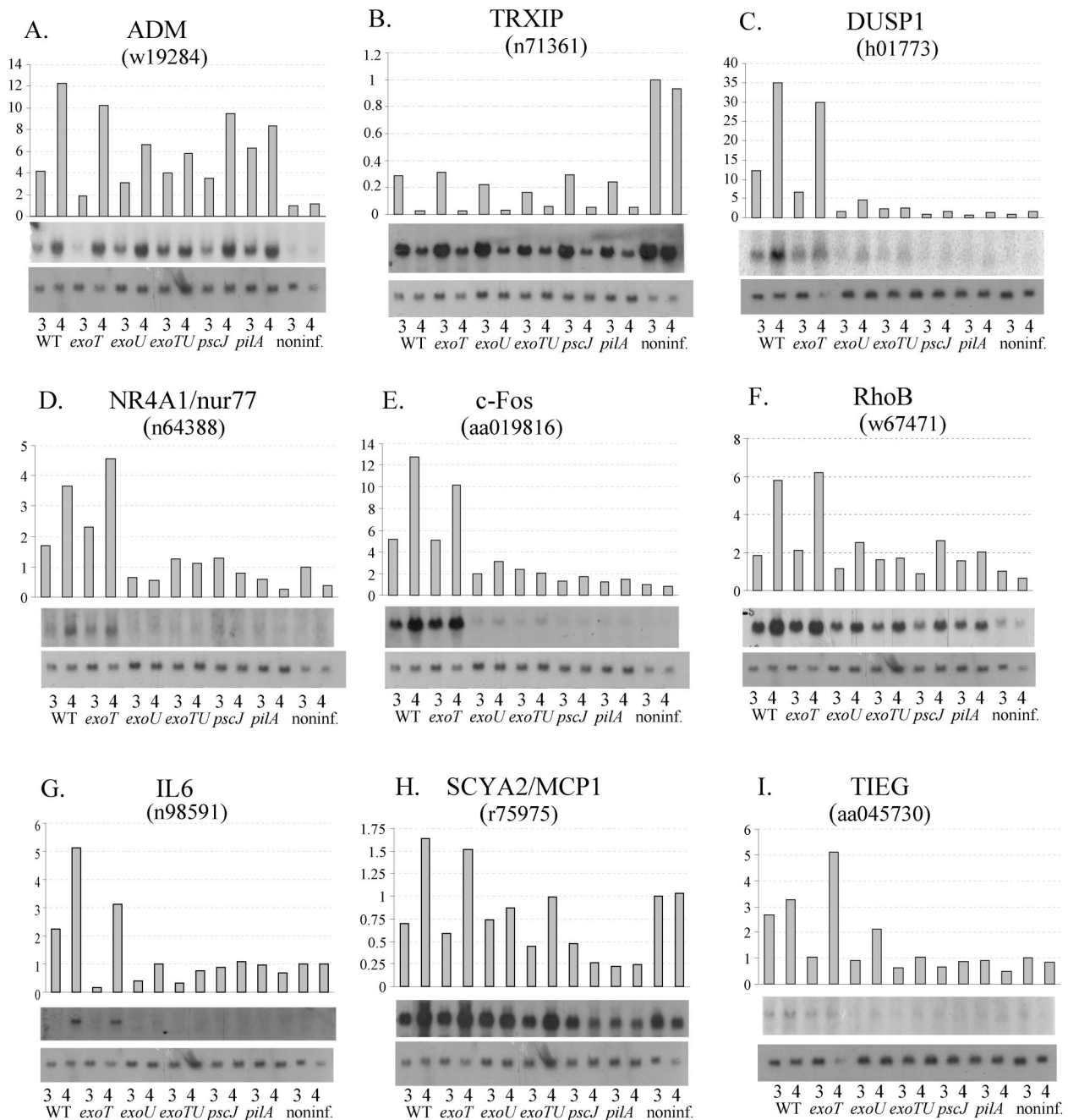


FIG. 3. Northern blot analysis of *P. aeruginosa* PA103 induced genes. Each panel depicts the quantified expression level relative to GAPDH expression (top), Northern blot autoradiograms of the specific gene (center), and the GAPDH loading control (bottom). Cells were infected with each indicated strain for 3 and 4 h. ADM and TRXIP are examples of class A and B genes, respectively. DUSP1, NR4A1, Fos, RhoB, and IL-6 are examples of class C genes, and SCYA2 and TIEG are examples of class D genes. GenBank accession numbers of clones from which the Northern probes were constructed are also noted.

(IL-6), a well-characterized inflammatory cytokine not represented on the array, was regulated in an ExoU-dependent manner (Fig. 3G). No ExoU-dependent genes were downregulated by infection.

The type III-dependent group (class D) contained two unique genes, MCP1/SCYA2 and transforming growth factor  $\beta$  (TGF- $\beta$ ) early inducible growth factor (TIEG), which encode known host inflammatory response proteins. Northern blotting

analysis of these two genes confirmed that they were regulated in the pattern inferred from the array analysis (Table 2 and Fig. 3H and I).

**ExoU-dependent induction of AP1 transcription factor activity.** A number of ExoU-dependent genes identified on the arrays are classified as immediate-early (IE) inducible genes (including c-Fos, c-Myc, DUSP1/MKP1, and RhoB), meaning that their regulation is subject to rapid changes in intracellular

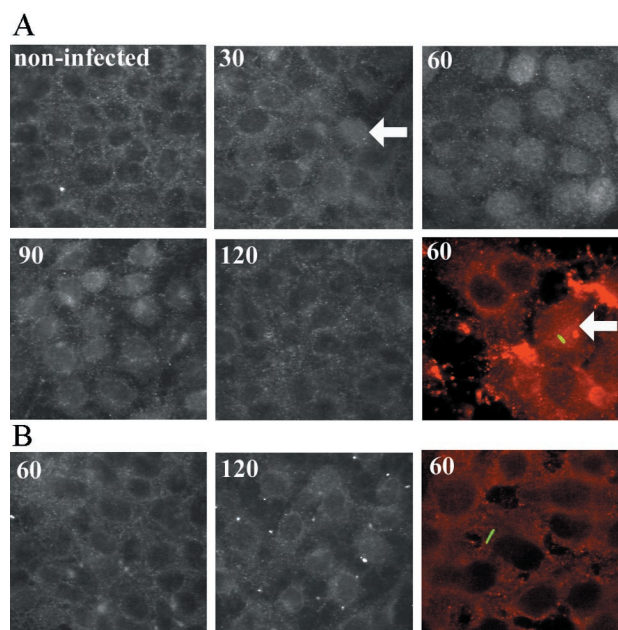


FIG. 4. Immunolocalization of c-Fos expression. Black and white panels represent images of cells immunostained with anti-c-Fos antibody after exposure to *P. aeruginosa* strains PA103 (A) or PA103 *exoU* (B) for different lengths of time as indicated (in minutes). Color panels represent images of cells doubly stained with anti-c-Fos antibody (red channel) and anti-*P. aeruginosa* antibody (green channel). Examples of nuclear localized c-Fos are indicated with arrows. Magnification,  $\times 600$ .

signaling and transcriptional activity rather than requiring de novo protein synthesis for their expression. Few of the type III-independent genes belong to this class, suggesting that ExoU was targeting a rapid response cell signaling pathway(s) during infection. Oncogene *c-fos* is one of the best-characterized IE genes. It is activated by a variety of stress-inducing stimuli and regulated via many signaling mechanisms including MAP kinase cascades. The c-Fos protein forms heterodimeric complexes with members of Jun protein family called AP1, which functions as a transcriptional activator of genes involved in cell protection and survival (65). Interestingly, other ExoU-dependent genes identified in the arrays were coregulated with *c-fos* in response to other stimuli, including *c-myc* (58) and DUSP1/MKP1 (62). In addition, NR4A1/nur77, SCYA2/MCP1, IL-6, and PLAUR are known to be activated by AP1 (7, 44, 46, 57). Therefore, c-Fos and AP1 may represent key mediators of the ExoU-stimulated response, and we decided to further characterize the mechanism of induction.

At the level of protein production, we observed no de novo increases in c-Fos protein during infection (data not shown). However, immunofluorescence studies revealed changes in the cellular location of c-Fos. The protein resides in the cytoplasm of quiescent cells but, upon stimulation, translocates to the nucleus, where it forms transcription factor complexes (50). In uninfected cells, significant levels of endogenous protein were localized to the cytoplasm (Fig. 4). Exposure to *P. aeruginosa* resulted in a rapid translocation of c-Fos to the nucleus (within 30 to 60 min), followed by the return to a cytoplasmic location by 120 min. This phenomenon was ExoU dependent since no

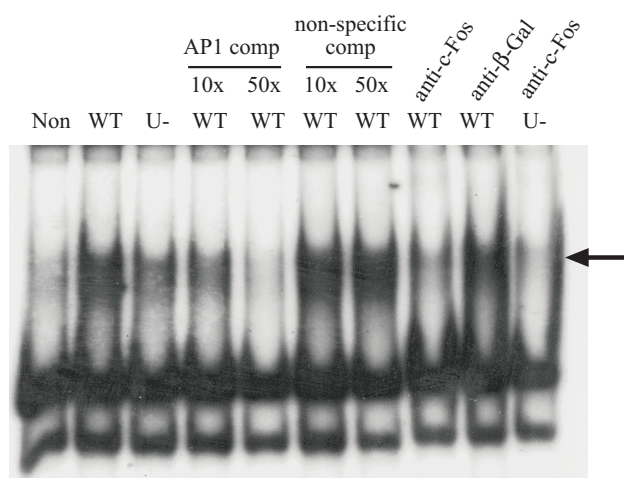


FIG. 5. AP1 EMSA. Nuclear extracts from noninfected cells (Non) and cells infected for 40 min with PA103 (WT) or PA103 *exoU* (U-) were incubated with radiolabeled AP1 oligonucleotide. An intense shifted band (arrow) indicates the presence of active DNA-binding AP1 complex. Competition experiments with 10- and 50-fold excess unlabeled (AP1 comp) AP1 oligonucleotide abolished the band, whereas nonspecific oligonucleotide (non-specific comp) did not, indicating the specificity of the DNA-protein complex. Inclusion of anti-c-Fos antibody, but not anti- $\beta$ -galactosidase (anti- $\beta$ -Gal) antibody, inhibited band formation, indicating that c-Fos protein was present in the active complex.

nuclear translocation was observed in cells exposed to the ExoU-deficient mutant. No further changes in protein location were observed after extended lengths of infection (up to 4 h) with either parental or mutant strains (data not shown). Using an anti-*P. aeruginosa* antibody, bacilli were observed interacting with cells at similar, but relatively low frequencies for both strains (estimated at 5 to 10% cells bound by bacteria), a finding consistent with observations in other polarized epithelial cell infection models (36). Although both bound and unbound cells contained nuclear c-Fos after exposure to the wild-type strain, no cells treated with the mutant strain (bound or unbound) were activated (Fig. 4), further suggesting that translocation required the function of ExoU.

The c-Fos protein functions as a DNA-binding transcription factor in a heterodimeric complex called AP1, the activity of which can be detected by using labeled oligonucleotides containing the AP1 recognition site in an EMSA. Active AP1 complex was not present in the nuclei of uninfected cells but was detected within 40 min of infection with the parental PA103 strain (Fig. 5). This coincided with the time at which c-Fos protein relocates from the cytoplasm to the nucleus. No AP1 activity was detected in cytoplasmic extracts (data not shown), and the AP1 complex contained c-Fos protein since anti-c-Fos antibody specifically abolished the AP1 band and caused a weak supershifted band (visible on extended exposure). AP1 activity was also detected after infection by ExoU-deficient bacteria. However, consistent with the other regulatory studies the band intensity was significantly less than wild-type levels, indicating the requirement of AP1 in the regulation of ExoU-dependent gene expression.



## DISCUSSION

Type III secretion allows for rapid contact and communication between bacteria and host cells. For many gram-negative species, the role of secreted type III effectors is to evade host defense mechanisms. In the case of *P. aeruginosa*, the effectors characterized to date appear to disable host cell actin cytoskeleton and cell-cell junctions, inhibit phagocytosis, and cause cell necrosis, thereby (presumably) disrupting the epithelial cell barrier and preventing an effective immune response. While much is understood about the functions of these effectors, little is known about the host cell response they may evoke. We sought to address this through the use of microarray studies that allowed the simultaneous measure of expression of thousands of different genes in conjunction with a controlled in vitro infection system and well-defined strains of *P. aeruginosa*. Our studies indicate the important role of type III secretion.

We detected a modest number of genes regulated in 9HTEo- cells by infection with *P. aeruginosa*. By comparison, macrophages may alter the expression of 10 to 20% of the total transcriptome in response to defined pathogen components such as lipopolysaccharide, and the response is idiosyncratic to particular pathogens (2). Other microarray studies have observed similarly small changes in gene expression in cultured lung epithelial cells infected with a different strain of *P. aeruginosa* (25) or in cytokine-stimulated lung epithelial cells (6). A comparison between our data and these earlier studies reveal similarly regulated genes, indicating that such microarray strategies are reproducible and comparable to findings of other labs. Using an alternative lung epithelial cell line (A549) and a different *P. aeruginosa* strain (PAK), which lacks flagella and secretes ExoS and ExoT but lacks ExoU, Ichikawa et al. (25) identified 22 genes that were upregulated after 3 h of *P. aeruginosa* exposure. Of the 15 represented on the microarrays used here, 5 were similarly induced. Other studies have addressed the response of epithelial cells to inflammatory stimuli such as tumor necrosis factor alpha and IL-1 $\beta$  exposure, and several genes are shared by these cells, including follistatin, stanniocalcin, TTP, MCP1/SCYA2 and vascular endothelial growth factor (VEGF) (6). This suggests the activation mechanism during *P. aeruginosa* exposure may involve the same signaling pathways as cytokines.

A major finding from the array study was the distinction between epithelial cell genes responsive to type III effectors and those regulated (presumably) by other bacterial factors. The latter set of genes fell into two expression classes: upregulated (class A) or downregulated (class B) by infection. It is interesting that there were no differences in the host cell transcriptional response to the type III secretion-defective mutant and the nonpilated mutant. This observation underscores three important points. First, the lack of host cell transcriptional response to the *pilA* mutant is not simply a consequence of its decreased binding compared to the *pscJ* mutant. Second, this finding suggests that the type IV pilus does not itself modulate the host cell transcriptional response, although it may affect host cell signal transduction. Finally, while some of the mutant strains tested are more efficiently internalized than others (i.e., the *exoU* *exoT* and *pscJ* mutants), no transcriptosome pattern specific to bacterial internalization could be identified.

Many of the genes responsive to factors other than the type III-secreted effectors are clearly candidates for roles in the early phase of inflammation induced by the organism. ADM is a peptide hormone that acts at sites of trauma to dilate the endothelium and permit the passage of inflammatory cells. It also possesses antimicrobial activity and has been identified in epithelial cells in response to other infectious stimuli (30, 60). VEGF has a range of biological activities, including endothelial cell migration, proliferation, and increasing vascular permeability (5, 17), and its production has recently been associated with epithelial infections (42, 45). Many other genes products upregulated by infection have roles in the regulation of infection/inflammation/stress response pathways. Follistatins regulate cytokine production in the acute phase response (8), RhoB is a negative regulator of NF- $\kappa$ B signaling (15), and insulin receptor substrate 2 (IRS2) mediates signaling from cytokine receptors (61). All of the known downregulated genes also have roles that fit with regulation of cellular response to infection. DUSP6/MKP3 is a specific inhibitor of the MAP kinase and extracellular regulated kinase (66), and MAP kinases are activated by *P. aeruginosa* infection (48). Particularly interesting was the downregulation of TRXIP. This molecule mediates the cell response to oxidative stress by regulating the expression and activity of thioredoxin (28). The fact that three metallothionein genes were also upregulated on the arrays suggests that redox maintenance and regulation in epithelial cells is an important process in the host response to infection, perhaps protecting the cell from the toxic free radicals produced by *P. aeruginosa*.

Approximately 50% of the genes regulated by *P. aeruginosa* infection were not affected by the type III secretion mutants. This suggests that the secreted effectors influence gene activation pathways that are distinct from other virulence factors. The largest group of such genes were induced only by strains producing ExoU. ExoU-deficient mutants are known to exert reduced cytotoxicity on cells (13, 21), but studies to date have so far failed to determine the exact mechanism of ExoU function (12). Our studies indicate that ExoU activity may result in AP1 transcription factor activation. Nuclear translocation of c-Fos and concomitant AP1 activation occurred rapidly (within 30 to 40 min) after exposure to the bacteria and required ExoU. Whereas c-Fos translocation occurred only in monolayers exposed to ExoU-sufficient strains, *P. aeruginosa*-bound cells, as well as unbound cells, were stimulated. Either binding is relatively weak at this time point, or biological activation by ExoU may involve cell-cell communication mechanisms.

A number of ExoU-dependent genes identified by array and expression studies are known to be AP1 responsive, including NR4A1/nur77, SCYA2/MCP1, and IL-6 (44, 46, 57). What are the possible signaling pathways that may link ExoU and AP1? Recent studies have shown that *P. aeruginosa* activates Ca<sup>2+</sup>-dependent MAP kinase signaling in a pilus-dependent manner (48). Several lines of evidence from our studies suggest that MAP kinase signaling is also involved in mediating ExoU-dependent expression. Some of the genes are MAP kinase responsive in other systems, including MCP1/SCYA2 (64), PLAUR (34), and *c-fos* and *c-myc* (59). DUSP1/MKP1 is also upregulated during MAP kinase activation and plays a negative feedback role by directly inactivating multiple MAP kinases (33). AP1 activity is regulated in part by phosphorylation

by the MAP kinase c-Jun N-terminal kinase (59). Therefore, ExoU may target components of a MAP kinase cascade that stimulate pathway activation or relieve its inhibition. Despite a lack of obvious cytotoxicity in the infected 9HTEo<sup>−</sup> cells during the infection time frame, the expression of several of the ExoU-dependent genes was consistent with an apoptotic and/or necrotic phenotype. NR4A1/nur77 has a proapoptotic role in T-cell development (43) and is upregulated after cytokine-induced growth arrest in melanoma cells (47). AP1 activity is involved in several apoptotic mechanisms (31). However, other ExoU-dependent gene products, such as DUSP1/MKP1 (14) and IRS2 (54, 67), have roles in protecting cells from apoptosis. Extended infection with *P. aeruginosa* (i.e., >24 h) does lead to cell death in 9HTEo<sup>−</sup> cells (E. O. Costelloe, unpublished data), and it is possible that these genes are induced in an attempt to delay the onset of apoptosis or necrosis.

The discovery of genes upregulated in an ExoU- and ExoT-independent, but type III-dependent manner (class D) suggests there are other secreted effectors in strain PA103 that can influence gene expression or that the type III secretion apparatus itself can activate host cell responses. Other studies have also raised this possibility (20). For example, the *exoU* *exoT* double mutant induces apoptosis in bone marrow-derived macrophages (Jakobsen and Engel, unpublished) and cytotoxicity when incubated with HeLa cells (16). However, the class D genes are likely to be markers of the host defense response rather than cytotoxicity in the 9HTEo<sup>−</sup> cells since no cytopathic effects were observed. MCP1/SCYA2 is a widely used marker of inflammatory action, and the product plays an important role in immune regulation by recruiting inflammatory cells to the site of infection (37). TIEG activation is indicative of TGF- $\beta$  function (27), which is important in the repair of wounded epithelial cells (23). Therefore, the identification of the molecule(s) responsible for this activity may provide new targets for the prevention of cell damage and antiinflammatory therapies in *P. aeruginosa* infection.

ExoT activity induces marked changes in the cytoskeletal structure and the internalization capacity of cultured HeLa cells and macrophages (16). However, in the 9HTEo<sup>−</sup> cells no effects attributable to ExoT were observed at the morphological or gene expression level (i.e., in comparing the ExoT-sufficient strain PA103 *exoU* with the ExoT-deficient PA103 *exoT* *exoU*, *pscJ*, and *pilA* strains). Although the reasons for this are not clear, it is possible that more polarized cells resist the effects of ExoT, since others have noted no phenotypic effects in confluent MDCK epithelial cells infected with an alternate PA103 *exoU* strain (13).

In conclusion, we have provided a here detailed account of the responses evoked by epithelial cells upon contact with *P. aeruginosa*; this is the first such study addressing the importance of type III-mediated toxin secretion in epithelial cell gene regulation. The host cell response can be dissected into toxin-dependent and -independent pathways, with MAP kinase cascades and AP1 activation being important components in the former. Further characterization of the pathways mediating type III toxin actions will help us to understand the potential benefits or problems associated with drugs that may be developed to inhibit type III secretion.

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